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Distinctly different behavioral responses of a copepod, *Temora longicornis*, to different strains of toxic dinoflagellates, *Alexandrium* spp.

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**Running head:** feeding behavior of copepod

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**Abstract**

Zooplankton responses to toxic algae are highly variable, even towards taxonomically closely related species or different strains of the same species. Here, the individual level feeding behavior of a copepod, *Temora longicornis*, was examined which offered 4 similarly sized strains of toxic dinoflagellate *Alexandrium* spp. and a non-toxic control strain of the dinoflagellate *Protoceratium reticulatum*. The strains varied in their cellular toxin concentration and composition and in lytic activity. High-speed video observations revealed four distinctly different strain-specific feeding responses of the copepod during 4 h incubations: (i) the ‘normal’ feeding behavior, in which the feeding appendages were beating almost constantly to produce a feeding current and most (90 %) of the captured algae were ingested; (ii) the beating activity of the feeding appendages was reduced by ca. 80 % during the initial 60 min of exposure, after which very few algae were captured and ingested; (iii) capture and ingestion rates remained high, but ingested cells were regurgitated; and (iv) the copepod continued beating its appendages and captured cells at a high rate, but after 60 min, most captured cells were rejected. The various prey aversion responses observed may have very different implications to the prey and their ability to form blooms: consumed but regurgitated cells are dead, captured but rejected cells survive and may give the prey a competitive advantage, while reduced feeding activity of the grazer may be equally beneficial to the prey and its competitors. These behaviors were not related to lytic activity or overall paralytic shellfish toxins (PSTs) content and composition and suggest that other cues are responsible for the responses.

## 1. Introduction

Zooplankton plays a crucial role in marine food webs, both by channeling primary production to higher trophic levels and by controlling phytoplankton populations. Algal blooms occur when algal growth exceeds zooplankton grazing. Thus, harmful algal blooms are thought to be facilitated by reduced grazing due to the algae producing toxic substances (Jonsson et al., 2009) that, in turn, are believed mainly to function as grazer deterrents. Reported grazer responses to harmful algae are

diverse. The responses of copepods, for example, to toxic algae may vary within and between species of both the grazers and algae, and responses range from unaffected to substantial (Turner, 2014). Even different populations of the same copepod species may show different responses to the same strain of a toxic alga due to acclimation or adaptation (Colin and Dam, 2002; Engström-Öst et al., 2002; Kozlowsky-Suzuki et al., 2003). A further complicating factor is that different strains and natural populations of the same algal species may vary in their toxicity and with its growth conditions (Burkholder and Glibert 2006; Cembella 1998). With a few exceptions (e.g., Hong et al. 2012), only macroscopic responses (e.g. mortality and feeding rate) rather than behavioral responses are examined, and in most cases it is not possible to establish a mechanistic relationship between the algal toxin profile and its effects on the copepod grazer.

The genus *Alexandrium* is found worldwide and is one of the most studied toxic dinoflagellates (Anderson et al., 2012). It includes 33 described species, of which 11 are known to produce paralytic shellfish toxins (PST) (Moestrup et al., 2009). The chemical structures of this group of toxins, including saxitoxin (STX) and approximately 57 derivatives, are well described from the genus and from seafood (Munday, 2014). Paralytic shellfish toxins are sodium-ion channel blockers that can cause potent neurotoxic syndromes in humans as well as fish, seabirds and marine mammals (Cembella, 1998; Turner and Tester, 1997; Turner, 2014). Reported effects on copepods offered PST-containing *Alexandrium* spp., however, range from none to adverse effects on ingestion rate, egg production, egg hatching and offspring development duration (Dutz, 1998; Frangopulos et al., 2000; Guisande et al., 2002). These variations in responses are not related to the overall toxicity of the cells (Teegarden et al., 2008) and raises the question of whether or not the PSP toxins actually function as a grazer deterrents. Could other compounds produced by *Alexandrium* spp. be responsible for the observed effects on copepods?

In fact, a number of different toxins have been found among *Alexandrium* spp. in addition to the PSTs, making interpretations of past reports difficult: spiroimines (spirolides, gymnodimines), goniodomin A and lytic compounds. The spiroimines are potent fast-acting neurotoxins that have so far only been found in the European and North Atlantic *A. ostenfeldii* but not Baltic *A. ostenfeldii* (Kremp et al., 2014; Sopanen et al., 2011). Goniodomin A is also a neurotoxin that has been reported to affect vertebrates (Klein et al., 2010) as well as invertebrates (Murakami et al., 1988). This toxin has only been reported for *A. hiranoi*, *A. monilatum* and *A. pseudogonyaulax* (Hsia et al., 2006; Murakami et al., 1998, 1988) and these species do not produce PSTs. Finally, many *Alexandrium* species and strains also have the ability to produce extracellular allelochemical compounds, which are still poorly examined chemically (Ma et al., 2009). These extracellular allelochemical compounds have been demonstrated to affect protistan grazers (Legrand et al., 2003; Tillmann and John, 2002), competitors (Granéli and Hansen, 2006; Legrand et al., 2003; Tillmann and Hansen, 2009), or paralyze prey cells (Blossom et al., 2012), while effects on metazoan grazers are still unknown. Thus, studies using experimental and control *Alexandrium* strains characterized as PST and non-PST strains might be misleading, as they may differ substantially in the presence/absence of other toxins/bioactive compounds.

Here, authors examined the initial behavioral response of the copepod *Temora longicornis* to 3 different strains of *Alexandrium tamarense*, a single strain of *A. pseudogonyaulax* and to a strain of *Protoceratium reticulatum* that contains no known toxins. Species and strains were selected due to their similar size and shape but different toxin content and profile (PSTs, lytic activity of the cells, Goniodomin A). Direct, high-speed video was used to describe feeding behaviors (activity, prey capture, rejection, ingestion, regurgitation). A wide prey-specific behavioral repertoire of the copepods were demonstrated that lead to a variation of ingestion rate and with distinctly different implications to the prey and their ability to form blooms. The behavioral response was unrelated to

the composition or content the compounds analyzed for the *A. tamarense* strains, suggesting that other compounds may trigger the avoidance behavior observed towards some of the prey.

## 2. Materials and methods

### 2.1 Algal cultures

A strain of *Protoceratium reticulatum* and 4 clonal strains of *Alexandrium* spp. were used in the experiments (Table 1). The culture of *P. reticulatum* CCMP1889 obtained from National Center for Marine Algae and Microbiota, *A. pseudogonyaulax* CAWD138 obtained from Cawthron Institute, and *A. tamarense* Alex2, *A. tamarense* Alex5, and *A. tamarense* AlexH5 obtained from Alfred Wegener Institute. The different algae were of similar size but varied in their toxin profiles (Table 1 and 2). Algal cultures were maintained on B1 medium prepared with pasteurized, filtered sea water at 16°C and a salinity of 32. The cultures were exposed to an irradiance of 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  on a 12 h: 12 h light: dark cycle. All phytoplankton used in the experiments were in exponential growth.

### 2.2 Toxin analyses

Paralytic shellfish toxins and lytic activity of the cells were quantified. Meanwhile, the presence of goniiodomin A, and yessotoxins (YTX) were tested. For cell content analyses, 10 to 20 ml of exponentially growing *Alexandrium* spp. and *P. reticulatum* cells (around 2000 cells  $\text{ml}^{-1}$ ) were centrifuged (2150 $\times$ g, 15 min). After removing most of the supernatant, the algae were re-suspended in 1 ml B1 medium and transferred to a micro-centrifuge tube. Samples were centrifuged again at 3200 $\times$ g for 15 min. All the supernatant was removed. The dry cell pellets were kept at -20 °C. Both cell concentrations of the initial algal culture and the supernatant were enumerated to calculate the exact number of cells in the pellets for toxin analysis.

Paralytic shellfish poisoning toxins (PSTs) were extracted with 500  $\mu$ l 0.03 mM acetic acid by ultrasonication (sonotrode HD 2070, Bandelin, Berlin, Germany; 1 min, cycle time 50%, 10 %power). The samples were centrifuged at 16,100 x g and supernatants filtered over centrifugation filters (pore-size 0.45  $\mu$ m, Millipore Ultrafree, Eschborn, Germany) at 1,500 x g for 30 sec. filtrates were transferred to autosampler vials and measured by ion-pair liquid chromatography coupled to post-column derivatization and fluorescence detection as described in detail in Suikkanen et al. (2013). In order to make the data comparable to other literature values, the combined cell toxicity was calculated as saxitoxin equivalents (STXeq) by multiplying toxin concentration values from HPLC chromatograms by toxin-specific toxicity equivalency factors (TEFs, Alexander et al. 2009) Since dinoflagellates are believed to exclusively produce the betamers of enantiomeric pairs (Cembella, 1998) and the corresponding alphamers are regarded as extraction artifacts, only TEFs of the betamers were used to calculate total toxicity as STX equivalents.

Lipophilic toxins (YTX and goniodomin A) were extracted with 300  $\mu$ l methanol and analyzed for YTX as described in detail in Sala-Pérez et al. (2016). All lipophilic extracts were also screened for two pseudomolecular ions of goniodomin A in the positive mode:  $m/z$  786 ( $[M+NH_4]^+$ ) and  $m/z$  791 ( $[M+Na]^+$ ) that were reported by Hsia et al. (2006). For the positive samples product ion spectra of both pseudomolecular ions were also recorded.

To quantify allelochemical (lytic) activity, the method of Blossom et al. (2014) was used, in which the concentration of dinoflagellate cells that cause 50 % mortality of target cells (*Teleaulax acuta*) is determined (Table 2 and Appendix Fig. 1). A target cell concentration of 3250 cells  $ml^{-1}$  and relative fluorescence were used to quantify target cell concentration. Vials with target cells and 10 to 15 different concentrations of supernatant obtained after centrifugation (2150xg, 15 min) of

dinoflagellate cultures were placed at 16°C in the dark for 3 h, at which time target cell survival was quantified fluorometrically (TD-700 Fluorometer, Turner Designs, San Jose, California, US).

### 2.3 Copepod feeding behavior

A feeding-current feeding copepod, *Temora longicornis*, were isolated from the Øresund, Denmark, and used to establish a continuous culture at 16°C, salinity 32. The culture was fed a mixed phytoplankton diet including *Akashiwo sanguinea*, *Heterocapsa triquetra*, *Prorocentrum minimum*, *Thalassiosira weissflogii*, and *Rhodomonas salina*.

Adult females used for video experiments were tethered to the dorsal surface with a short length of human hair using a small drop of super glue (Cowles and Strickler, 1983) and placed overnight in filtered sea water in a dark, thermo- constant room (16°C). The subsequent morning, the other end of the hair was attached to a micromanipulator and the copepod was placed in a 10×10×10 cm<sup>3</sup> transparent container filled with filtered sea water in a thermo- constant room. The tethered copepods may live for many days and appear unaffected by the tether.

Phytoplankton was added at time 0, and the behavior of the copepod recorded during the subsequent 4 hours. Copepods were offered one of the four strains of *Alexandrium* spp. or *P. reticulatum* at one of 3 different concentrations (40, 80 and 200 cells ml<sup>-1</sup>;  $\pm <10\%$ ). Three individual copepods were tested for each strain/concentration treatment, totaling 9 individuals per strain. Samples (3 ml) for algal enumeration were removed during the beginning, middle and end of filming to check the prey concentrations. Also, the water was gently stirred throughout the experiment to prevent sedimentation of the algae. The tethered copepod was filmed with a Phantom V210 high speed camera using infrared illumination shined through the aquarium towards the camera. The camera was equipped with Nikon lenses to yield a field of view of approximately 2.5×1.6 mm<sup>2</sup> (varied slightly between experiments). Both high speed (resolution: 1280×800 pixels; frame rate: 2200 Hz)



and low speed (resolution: 720×576 pixels; frame rate: 25 Hz) videos were saved simultaneously from the camera. The low speed video was set to save automatically the first 30 minutes and then for 10 minutes every ½ hour to describe feeding activity and prey interactions. Several 2.5 sec sequences of high speed recordings were saved through the entire experimental duration to quantify appendage beat frequencies and describe prey response behaviors.

The feeding current of *T. longicornis* is created by the regular beating of the second antenna (A2) and the maxillipeds (MXP) as well as of the other feeding appendages (Gonçalves et al., 2014; Paffenhöfer et al., 1982; Tiselius et al., 2013) (Appendix Video 1). When a prey particle within the feeding current touches the setae on one of the feeding appendages and is detected, the regular beating of the feeding appendages is changed to guide the prey particle next to mandibles. An event as a ‘capture’ was classified when the prey particle was handled by the copepod (Appendix Video 1). After being captured, the prey was generally handled for a short period and adjusted to a certain position before either being swept into the mouth, an ‘ingestion’ event (Appendix Video 1), or being rejected (a ‘rejection’ event; Appendix Video 2). In some cases, all or parts of a prey particle were regurgitated after ingestion, which was recorded as both ‘ingestion’ and ‘regurgitation’ events (Appendix Video 3).

The low speed recordings were used to enumerate capture, ingestion, rejection, and regurgitation events and to quantify the fraction of time the animal was beating its feeding appendages. The fraction of time beating was estimated by counting the number of frames that the copepod was beating its appendages during the last 1 minute of every 10 minutes sequence.

The high speed video was used mainly to quantify the beat frequency of the appendages. Characteristic sequences were also saved to illustrate the various types of feeding behaviours (Appendix Video 1-4). Prey positions and beating frequencies were measured using ImageJ

(Version 1.48; National Institutes of Health, USA) and Phantom Cine Viewer (Version 2.6; Vision Research).

Waterborne cues from copepod grazers can induce increased PSTs production in *Alexandrium* spp. (Selander et al., 2006), but the full induction takes 2-4 days (Selander et al., 2012), and is low with the low concentration of copepods used here (1 per 800 mL). Thus, it is assumed that the chemical profile of cells from the culture is representative for the experiments.

## 2.4 Statistical analysis

Differences in appendage beat frequency, fraction of time beating, capture rate, ingestion rate, and fraction of captured cells rejected between treatments were tested using two-way ANOVA with prey concentration and prey species as factors. Mean values were compared using Holm-Sidak Test and carried out in SigmaPlot 13.0. Normality was tested according to Shapiro-Wilk.

## 3. Results

### 3.1 Algal toxin content

6 different species of PSTs were identified (Table 2). Strain Alex5 contained mainly C1/C2, GTX 1/4, STX and NEO. AlexH5 also had high cell toxin content, but mainly C1/C2 and GTX 1/4; it lacked STX. Strain Alex2 had fewer PST derivatives and an order of magnitude lower cellular PST content. A compound with the molecular mass of goniodomin A was only detected in *A. pseudogonyaulax*, which did not have PSTs. The strain of *P. reticulatum* contained neither YTX nor other toxins (lytic compounds, PSTs or goniodomin A) above detection level and hence worked as a non-toxic control.

2 strains of *A. tamarense* (Alex2 and AlexH5) both produced and excreted compounds with lytic effects on the test organism *T. acuta* (Table 2).

### 3.2 Appendage beat frequency

The cephalic appendages of *T. longicornis* produce a continuous repetitive beating. The appendage beat frequency varied between 22 and 34 Hz between the 5 diets (Fig. 1A). The variation was independent of prey concentrations ( $P=0.905$ ), and time ( $P=0.380$ ; data in Appendix Fig. 2), but differed significantly between prey ( $P<0.05$ ). The beat frequency was highest (33 Hz) when fed on *A. tamarens* AlexH5 at all prey concentrations, while the beat frequencies of copepods exposed to the other four preys were similar to one another and averaged 26 Hz. Time-resolved patterns in beat frequencies are given in Appendix Fig. 2.

### 3.3 Fraction of time the feeding appendages beat

Initially all the copepods were using their appendages constantly. Most of them kept beating at near 100% of the time during all 4 observation hours (Appendix Video 1-3), except copepods exposed to *A. tamarens* Alex5 (Appendix Video 4). With this prey, the beating activity of the copepods decreased rapidly during the first hour to reach about 20 % of the time and then remained at that level during the remaining 3 h (Fig. 1B and 2A). The decline was statistically significant ( $p < 0.05$ ) but independent of prey concentration ( $P=0.222$ ). Since several aspects of the feeding behavior changed during the first hour but subsequently remained relatively stable, all the statistical analyses below consider only the last 3 h of each experiment. Time resolved patterns in appendage activity are shown for all prey in Appendix Fig. 3.

### 3.4 Capture rate

Prey capture rate increased with increasing prey concentration for all prey types (Fig. 1C, time resolved in Appendix Fig. 4) and differed significantly between prey species and concentrations ( $P<0.05$ ). With the same prey concentration, *A. pseudogonyaulax* were captured at the highest rate

and *A. tamarensis* Alex5 at the lowest rate. The other strains were captured at intermediate and similar rates.

### 3.5 Rejection

Captured prey may be ingested or rejected. Initially, all copepods rejected only a small fraction ( $\approx 20\%$ ) of captured cells. After 60 minutes, the proportion of rejected *A. pseudogonyaulax* cells increased to  $\approx 80\%$  and remained at this level till the end of the observation period (Fig. 1D and 2B, Appendix Video 2, time-resolved pattern in Appendix Fig. 5). With the other four prey strains the fraction of rejected cells remained stable and low (Appendix Fig. 5). Thus, not including the first hour, the fraction of rejected cells was significantly higher with *A. pseudogonyaulax* ( $0.9 \pm 0.1$ ) compared to the four other prey strains ( $0.2 \pm 0.1$ ) ( $p < 0.05$ ), while prey rejection was independent of prey concentration for all five prey ( $P=0.152$ ).

### 3.6 Ingestion rate

The ingestion rate of prey is the product of capture rate and the fraction of accepted (i.e., not rejected) cells. The ingestion rate increased with the increasing of prey concentration with all prey ( $p < 0.05$ ), and there were also significant differences between prey strains ( $P < 0.05$ ) (Fig. 1E). Non-toxic control, *P. reticulatum*, cells were consistently ingested at the highest rate ( $80 \pm 60$  cells  $\text{h}^{-1}$  at  $200$  cells  $\text{ml}^{-1}$ ), and *A. pseudogonyaulax* and *A. tamarensis* Alex5 ( $17.1 \pm 7.3$  and  $23.1 \pm 8.0$  cell  $\text{h}^{-1}$  at  $200$  cells  $\text{ml}^{-1}$ ) at the lowest rates, with the other strains in between (time resolved in Appendix Fig. 6).

### 3.7 Regurgitation

Some ingested cells were rapidly (within 1 s) regurgitated. This was in particular evident with *A. tamarensis* Alex2 as prey (Appendix Video 3). The proportion of cells regurgitated increased with

increasing *A. tamarense* Alex2 concentration to more than 30 % at the highest concentration (Fig. 1F, Appendix Fig. 7), but was independent of time ( $P=0.670$ ). A small proportion (2% and 13%) of ingested cells was also regurgitated when copepods were fed a high concentration of *A. tamarense* AlexH5 and *A. pseudogonyaulax*, respectively.

### 3.8 Ingestion of PSTs by the copepods

Based on the ingestion and regurgitation of phytoplankton cells by copepods (Fig. 1) and the toxin content of each algal prey (Table 2), the total ingestion of PSTs (in STX equivalents) and STX on the three diets of *A. tamarense* were calculated (Fig. 3, Appendix Fig. 8-10). The cumulated amount of ingested PSTs increased over time and with cell concentration, and was highest in copepods offered AlexH5, and lowest when offered Alex2 (Fig. 3A). Due to a lack of STX in Alex H5, The highest accumulation of STX was in copepods offered Alex5 and followed by copepods offered Alex2 (Fig. 3B).

## 4. Discussion

### 4.1 Repertoire of copepod feeding behaviors and implications to prey populations

4 distinctly different behavioral responses of the copepod were observed to various prey cells, viz.: (i) normal feeding behavior - the feeding appendages are beating more or less constantly and most captured cells are ingested (control alga *P. reticulatum* and AlexH5); (ii) the copepod significantly reduces the fraction of time it is beating its appendages in the course of the first hour after introducing prey cells and beating activity then remains low; captured cells are however mainly ingested, although at a low rate (Alex5); (iii) appendage beat activity remains high and cells are captured and ingested at a high rate, but a large fraction of the ingested material is subsequently regurgitated (Alex2); and (iv) feeding activity and prey capture rate remains high, but an increasing fraction of captured cells are rejected during the first hour, and rejection rate remains high during

the remainder of the observation period (*A. pseudogonyaulax*). Most previous studies of the response of copepods to toxic algae are incubation studies, in which the net outcome of the copepod-prey interaction is quantified in terms of feeding rate, prey selection, growth or egg production rate, or other similar bulk measures (reviewed by Turner 2014). The direct video observation of individual responses and of direct copepod-prey cell interactions provided by this and a few other studies (Bruno et al., 2012; Hong et al., 2012; Tiselius et al., 2013) are innovative and allow us to disentangle the possible mechanisms underlying the diverse outcome of ‘black box’ incubation experiments and to better evaluate their ecological significance.

Several studies have reported that copepods may select between toxic and non-toxic cells in a prey mixture (DeMott and Moxter, 1991; Huntley et al., 1986; Schultz and Kiørboe, 2009; Selander et al., 2006; Teegarden, 1999). The fact that the copepods can distinguish between cells of very similar size and shape suggests that selection is mediated by chemical information. Schultz and Kiørboe (2009) suggested that copepods possess the ability to remotely discriminate non-toxic and toxic algae before capture. Recently however the ability of copepods to remotely detect phytoplankton based on their chemical characteristics has been questioned (Gonçalves and Kiørboe, 2015), and our observations suggest that prey selection is based on post-capture discrimination and that unwanted cells are rejected following a handling time. Vanderploeg et al. (1990) reported a similar observation in a freshwater copepod. Thus, prey selection appears to be based on gustation (taste) rather than olfaction (smell).

Multiple studies have reported reduced feeding rates on toxic compared to similarly sized and shaped non-toxic algae in single-prey experiments (Turner, 2014), and our observations suggest two possible mechanisms behind such a response, i.e., rejection of captured cells before ingestion and reduced feeding activity. An increased cell rejection was observed only with *A. pseudogonyaulax* as prey, and the increasing rejection rate during the first hour, suggesting that the copepod would need

to learn that these cells are un-wanted. The reduced feeding activity response (reduced appendage beat activity) was only observed with *A. tamarense* Alex5 as food. Since the lytic compounds detected from Alex H5 had no effect on the feeding behavior of *T. longicornis*, the extracellular compounds were not the trigger. The reduced feeding activity only materializes after the copepod has ingested some cells, and so is likely mediated by substances released during processing of food in the gut. Subsequent to this transition period the copepod keeps beating its feeding appendages intermittently and captures and ingests prey cells at a low rate, allowing the copepod to continuously sample the environment and – presumably – to pick up feeding at high rate if the prey environment changes. A similar behavior is observed in the copepod *Acartia tonsa*. This copepod modifies its appendage beat activity and feeding current production in response to the concentration and type (size, motility) of prey cells in the environment (Jonsson and Tiselius, 1990), allowing it to switch between feeding current feeding and ambush feeding. In the presence of toxic *Karenia* spp. cells it also (within 10 min) reduces appendage beat activity to only sample the environment, and resumes more active feeding if the prey environment becomes favorable (Hong et al., 2012).

Some studies have demonstrated reduced growth and egg production rates (Colin and Dam, 2007; Dutz, 1998; Guisande et al., 2002; Roncalli et al., 2016; Sopanen et al., 2011; Teegarden et al., 2008) or elevated mortality (Avery et al., 2008; Sopanen et al., 2011) in copepods exposed to toxic algae compared to control algae. Such responses may be mediated by the behaviors considered above that both lead to reduced prey ingestion, or by the regurgitation of consumed algae, as described here for *T. longicornis* feeding on *A. tamarense* Alex2. From our video observations, most of the regurgitated cells were smashed and so it is impossible to quantify the exact amount of food lost through regurgitation, but it may be significant. Sykes and Huntley (1987) reported a similar observation of the copepod *Calanus finmarchicus* regurgitating *Protoceratium reticulatum* (= *Gonyaulax grindleyi*), and found that the copepod was unable to fill its gut, suggesting a

significant reduction in net food intake. While the regurgitation response observed by Sykes and Huntley only occurred 45-120 min after initiation of feeding, the response reported here is immediate and specific to the cell just consumed.

All the prey aversion responses observed here, viz. reduced feeding activity, rejection of captured cells, and regurgitation of ingested cells, may all lead to reduced energy uptake, growth, and egg production of the copepod. The ecological and evolutionary implications of the responses differ in several important ways. First, a prey cue that leads to prey rejection, that is, a true feeding deterrent, is beneficial to the algal cell as it survives the interaction with the copepod, allows the copepod to continue to feed on competing but palatable cells, and may lead to the formation of a bloom. It is also easy to envisage how such feeding deterrent can evolve as it gives the individual cell a competitive advantage. Second, a cue that leads to reduced feeding activity, although beneficial to the compounds producer, may be equally beneficial to its competitors, and cheaters that do not pay the price of compound production may flourish. It appears not to be an evolutionary stable strategy and may not lead to the formation of a bloom of the compound producer. Thirdly, an ingested but regurgitated cell is not beneficial to the individual cell (it is dead), and although it may reduce grazing due to reduced growth or survival of the predators, it does so only on its sibling cells and equally on its competitors.

#### **4.2 Potential role of cellular toxin quantity and composition on copepod behavioral responses**

It is unclear from our results and from data in the literature exactly what elicits the very different behavioral responses in copepods exposed to the various strains of *Alexandrium*, except that it most likely is a chemical cue contained in or released by the cells.

With respect to PSTs, their mode of action in vertebrates is known to be a binding to voltage-gated sodium channels inhibiting action potential, nerve transmission, and ultimately muscle contraction



(Cusick and Sayler, 2013), and it thus could be expected that voltage-gated sodium channels of invertebrates are likewise affected. Although a number of invertebrates retain and accumulate PSTs in their tissues, many species including a number of bivalve mollusks are – contrary to popular belief - not immune to PSTs (Gaines and Shumway, 1988; Kvitek and Beitler, 1991; Robineau et al., 1991). Paralytic shellfish toxins resistance in soft shell clams has been identified to be caused by a single mutation in the saxitoxin binding site in the sodium channel (Bricelj et al., 2005). Copepods have also been demonstrated to adapt to PSTs and become immune after some generations of exposure (Colin and Dam, 2007, 2004), but the mechanism of adaptation remains unknown. Like in Bivalves, sodium channel mutants have been identified in the copepod *A. hudsonica*, but these turned out not to account for achieved immunity in this copepod species (Finiguerra et al., 2015).

Considering the toxin composition in the PSTs containing strains used in our experiments, the most distinct response (in terms of ingestion rates) is found when fed on Alex5 (reduced beating activity) followed by Alex2 (regurgitation) and with no response to AlexH5. This pattern of behavioral responses cannot be easily explained by cellular content or ingestion of total PSTs (in STXeq) (Table 2 and Fig. 3A). AlexH5 among all PST-producing strains tested had the highest cellular toxin content, both as total compounds per cell and when calculated as STX equivalents (Table 2), but failed to cause obvious copepod behavioral responses. The common conversion of all quantities of single congeners to one estimate of total toxicity (in STX eq) is justified by how toxic they are to humans, and thus Toxicity-Equivalent-Factors based on the standard mouse bioassay (Munday, 2014) are used. It can be a reasonable assumption that relative potency of each single PST compound may vary dramatically for different sodium channel types even for vertebrate cells (Alonso et al., 2016) and it is unknown whether and how invertebrate sodium channels are differentially affected. Thus, copepod behavior might be related to single toxin compounds, and copepod behavior was indeed correlated with cell content and total ingestion of the STX molecule

(Fig. 3B), suggesting that specifically STX plays an important role for the observed copepod behavioral changes. Such a view, however, is likely too simplistic, as all *A. tamarense* strains also had significant amounts of the nearly identical molecule neosaxitoxin (NEO). While Alex5 (reduced beating activity) also had the most STX and NEO when combined, AlexH5 (no effect) had more than Alex2 (regurgitation). So the observed behavior cannot be related to estimates of total STX+NEO ingestion (see Appendix fig. 8-10). The inconclusive pattern of behavioral responses in our experiments aligns well with the results of the incubation experiments of (Teegarden et al., 2008): the very different feeding rates in 4 different species of copepods to *Alexandrium* spp. strains of different PST toxicity that they observed were unrelated to the level of total toxicity (as STX eq per cell) and only related to whether or not the cells were toxic. Thus, the resolution of the feeding response into the more diverse behavioral responses and the information on the composition of toxins reported here do not appear to provide clear answers to the identity and nature of the cues that are responsible for the responses.

The presence of lytic compounds also did not correlate with behavioral changes of *T. longicornis*. Allelochemicals from *Alexandrium* are assumed to primarily act destructively on the external plasma membrane and have been shown to have a high lysis potential for single protistan cells (Tillmann et al., 2008). Authors here for the first time provide evidence that these lytic compounds, at least at the concentrations applied in our experiments, do not affect the short term feeding behavior of *T. longicornis*.

The response of the copepod to one strain of *A. pseudogonyaulax* was also examined. This species has been described to produce the neurotoxin goniiodomin A, but no PSTs. The *A. pseudogonyaulax* strain used in this study contained a dominant peak of a compound with the mass of goniiodomin A and was the only strain that elicited strong prey rejection responses.

In conclusion, the higher resolution of the behavioral responses revealed by direct observations compared to incubation approaches has demonstrated a high degree of strain-specificity, not only in bulk grazing reduction but also in how grazing reduction is achieved. Our comparative approach of using a number of *Alexandrium* strains, which are considered toxic from a human health point of view, differing in the amount and type of toxins was successful in providing first evidence that goniiodomin A plays a role as a true grazer deterrent. Moreover, there is no evidence that lytic compounds affect *T. longicornis* feeding behavior. On the other hand, behavioural response of *T. longicornis* to three PST-producing strains, even when acknowledging their differences in total amounts and PST profile, was too different to accept a universal role of PSTs in affecting *T. longicornis* feeding, and other substances may provide the cues for the diverse behavioral responses observed here. One promising avenue to pursue may be to combine directly observed responses with metabolic profiling of the phytoplankton as applied to resolve other plankton chemical cues (Selander et al., 2016).

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Fig. 1. Feeding behaviors of *Temora longicornis* fed on *Protoceratium reticulatum*, *Alexandrium tamarense*, and *A. pseudogonyaulax* at three prey concentrations (40, 80 and 200 cells ml<sup>-1</sup>). N=3, error bars represent standard deviation. (A) Average beating frequency (Hz) of *T. longicornis* during all four hours of video experiments. (B) Average fraction of time when *T. longicornis* was beating its feeding appendages during the last three hours of video experiments. (C) Average prey cells observed to be captured per copepod per hour during the last three hours of video experiments. (D) Average fraction of prey cells that was rejected after being captured by *T. longicornis* during the last three hours of video experiments. (E) Average prey cells observed to be ingested per copepod per hour during the last three hours of video experiments. (F) Average fraction of prey cells that was regurgitated after being ingested by *T. longicornis* during the last three hours of video experiments.

Fig. 2. Temporal behavioral variation of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments. (A) The fraction of time beating of *T. longicornis* fed on *A. tamarense* (Alex5) decreased independently of prey concentration. (B) The fraction of rejected of *T. longicornis* fed on *A. pseudogonyaulax* increased independently of prey concentration.

Fig. 3 The cumulative ingested PSTs in *Temora longicornis* when fed *Alexandrium tamarense* strains: Alex5 (solid line), Alex2 (dashed line), and AlexH5 (dotted line). The cumulative total ingested PST by copepods exposed to prey concentrations of 40 cells ml<sup>-1</sup>(A-1), 80 cells ml<sup>-1</sup>(A-2), and 200 cells ml<sup>-1</sup>(A-3). The



cumulative ingested saxitoxin (STX) at prey concentrations of 40 cells ml<sup>-1</sup>(B-1), 80 cells ml<sup>-1</sup>(B-2), and 200 cells ml<sup>-1</sup>(B-3). Values are means (n=3).

#### Online figures

Appendix fig. 1. Variation in lytic activity among different strains of prey algae by showing the mortality of the target cell, *Teleaulax acuta*.

Appendix fig. 2. The beating frequency of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix Fig. 3. The fraction of time beating of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix fig. 4. The capture rate of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix Fig. 5. The fraction of rejected of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix Fig. 6. The ingestion rate of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix Fig. 7. The fraction of regurgitated of *Temora longicornis* fed *Alexandrium* spp. during the four hours video experiments.

Appendix Fig. 8. The cumulative ingested PSTs in *Temora longicornis* when fed 40cells ml<sup>-1</sup> of *Alexandrium tamarense* strains: Alex5 (solid line), Alex2 (dashed line), and AlexH5 (dotted line).

Appendix Fig. 9. The cumulative ingested PSTs in *Temora longicornis* when fed 80cells ml<sup>-1</sup> of *Alexandrium tamarense* strains: Alex5 (solid line), Alex2 (dashed line), and AlexH5 (dotted line).

Appendix Fig. 10. The cumulative ingested PSTs in *Temora longicornis* when fed 200cells ml<sup>-1</sup> of *Alexandrium tamarense* strains: Alex5 (solid line), Alex2 (dashed line), and AlexH5 (dotted line).

#### Online Videos

Appendix Video 1: *Temora longicornis* beats regularly and captures a prey cell (*Alexandrium tamarense* AlexH5) suddenly. After handling a short period, the cell is ingested. Video sequence is 20 times slower than real speed.

Appendix Video 2: *Temora longicornis* beats regularly and captures a prey cell (*Alexandrium pseudogonyaulax*) suddenly. After handling and tasting, the cell is rejected by the copepod. Video sequence is 20 times slower than real speed.

Appendix Video 3: *Temora longicornis* regurgitates some crush after eating a prey cell (*Alexandrium tamarense* Alex2). Video sequence is 20 times slower than real speed.

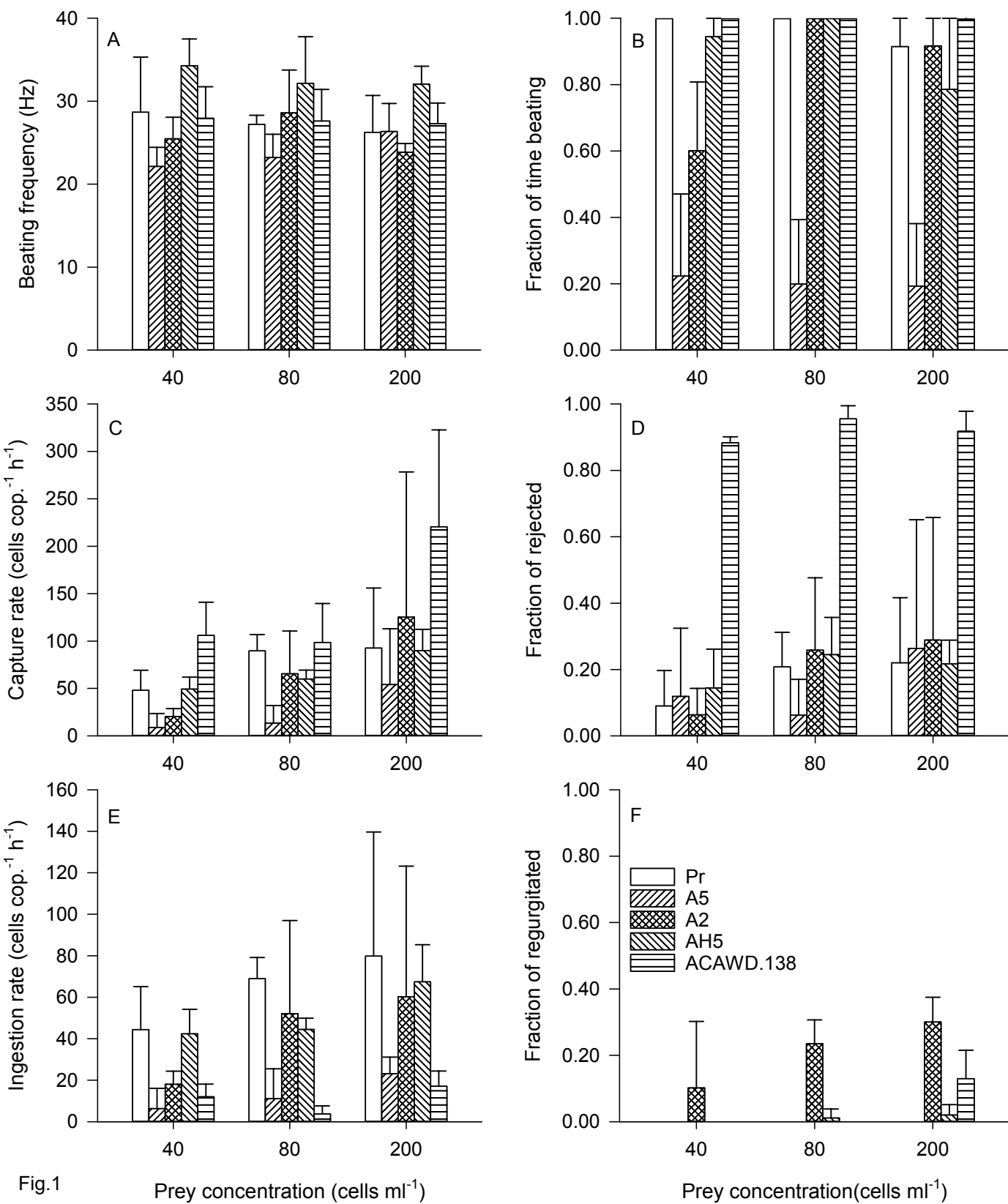
Appendix Video 4: *Temora longicornis* beats irregularly when exposed in *Alexandrium tamarense* Alex5. Both beating frequency and fraction of time beating decrease. Video sequence is 20 times slower than real speed.

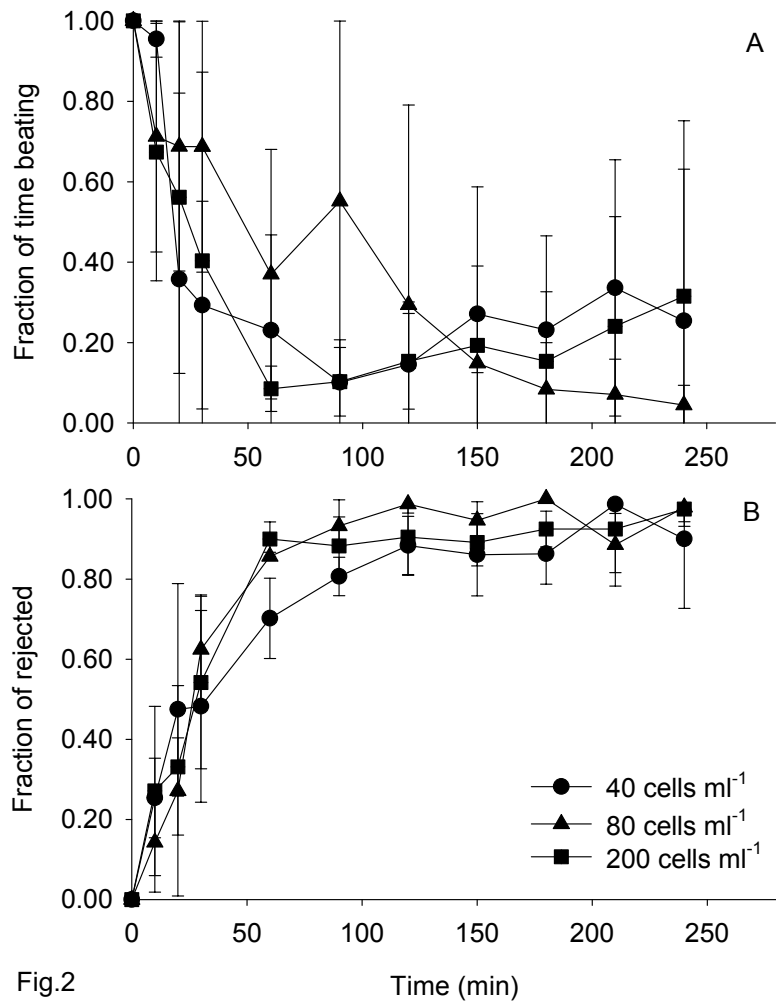
Table 1. List of the algae used as prey species for *Temora longicornis* in video observations, including the strain number, the isolation location, and the equivalent spherical diameter (ESD).

Algae	Strain	Origin	ESD ±SD (µm)	Reference
<i>Protoceratium reticulatum</i>	CCMP1889	Friday Harbor, USA	32.0±2.3	(Howard et al., 2009)
<i>Alexandrium tamarense</i>	Alex2	North Sea off Scotland	31.3±2.5	(Tillmann and Hansen, 2009; Tillmann et al., 2009)
	Alex5	North Sea off Scotland	33.8±0.5	(Tillmann and Hansen, 2009; Tillmann et al., 2009)
	AlexH5	Gulf of San Jorge, Argentina	31.6±0.7	(Krock et al., 2015)
<i>Alexandrium pseudogonyaulax</i>	CAWD138	Kerikeri, New Zealand	33.8±0.9	

Table 2. Toxin profiles and contents of the algae. PST = paralytic shellfish toxins; YTX = Yessotoxin; GA = Goniiodomin A; LA = Lytic activity. + = toxin detected but not quantified; - = not detected.

Algae	Strain	PSTs (fmol cell <sup>-1</sup> )							Cell Toxicity (pg STXeq cell <sup>-1</sup> )	YTX	GA	LA LC <sub>50</sub> (cells ml <sup>-1</sup> )
		C1/C2	GTX1/4	dcGTX2/3	GTX2/3	NEO	STX	total				
<i>Protoceratium reticulatum</i>	CCMP1889	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-
<i>Alexandrium tamarense</i>	Alex2	4.7	0.0	0.0	0.4	4.2	2.1	11.4	2.3	-	-	511
	Alex5	43.0	40.6	2.3	3.9	27.5	10.8	128.1	29.1	-	-	-
	AlexH5	119.5	40.1	0.1	2.9	8.5	0.0	171.0	22.9	-	-	544
<i>Alexandrium pseudogonyaulax</i>	CAWD138	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	+	-





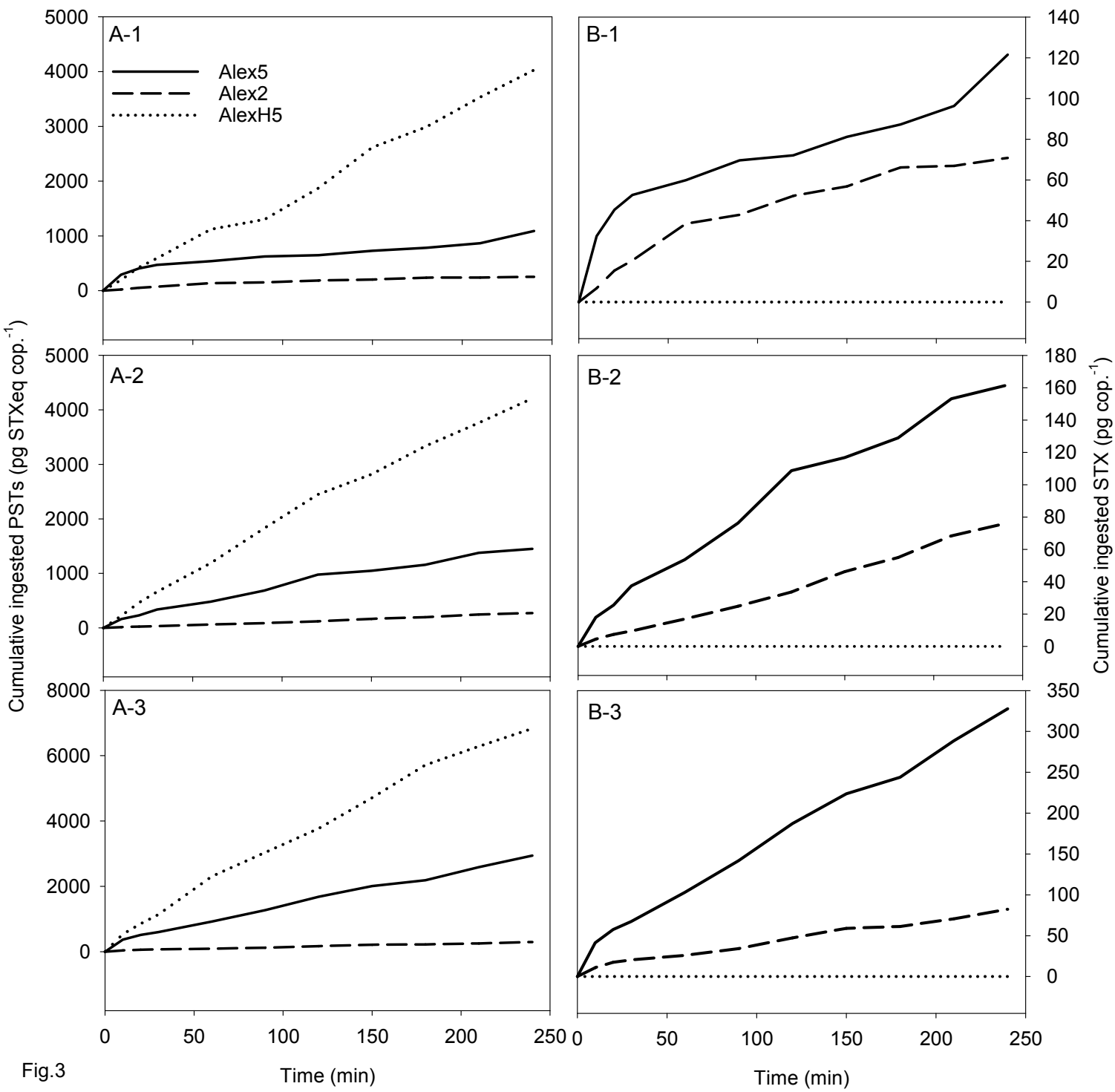
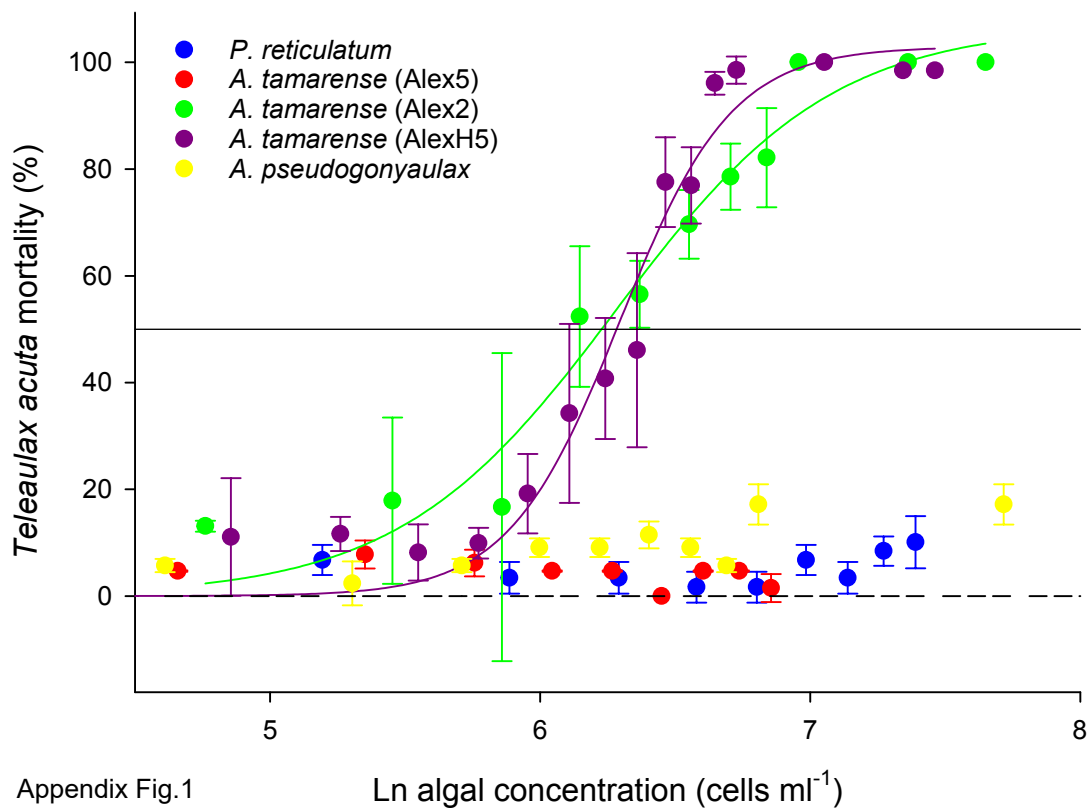
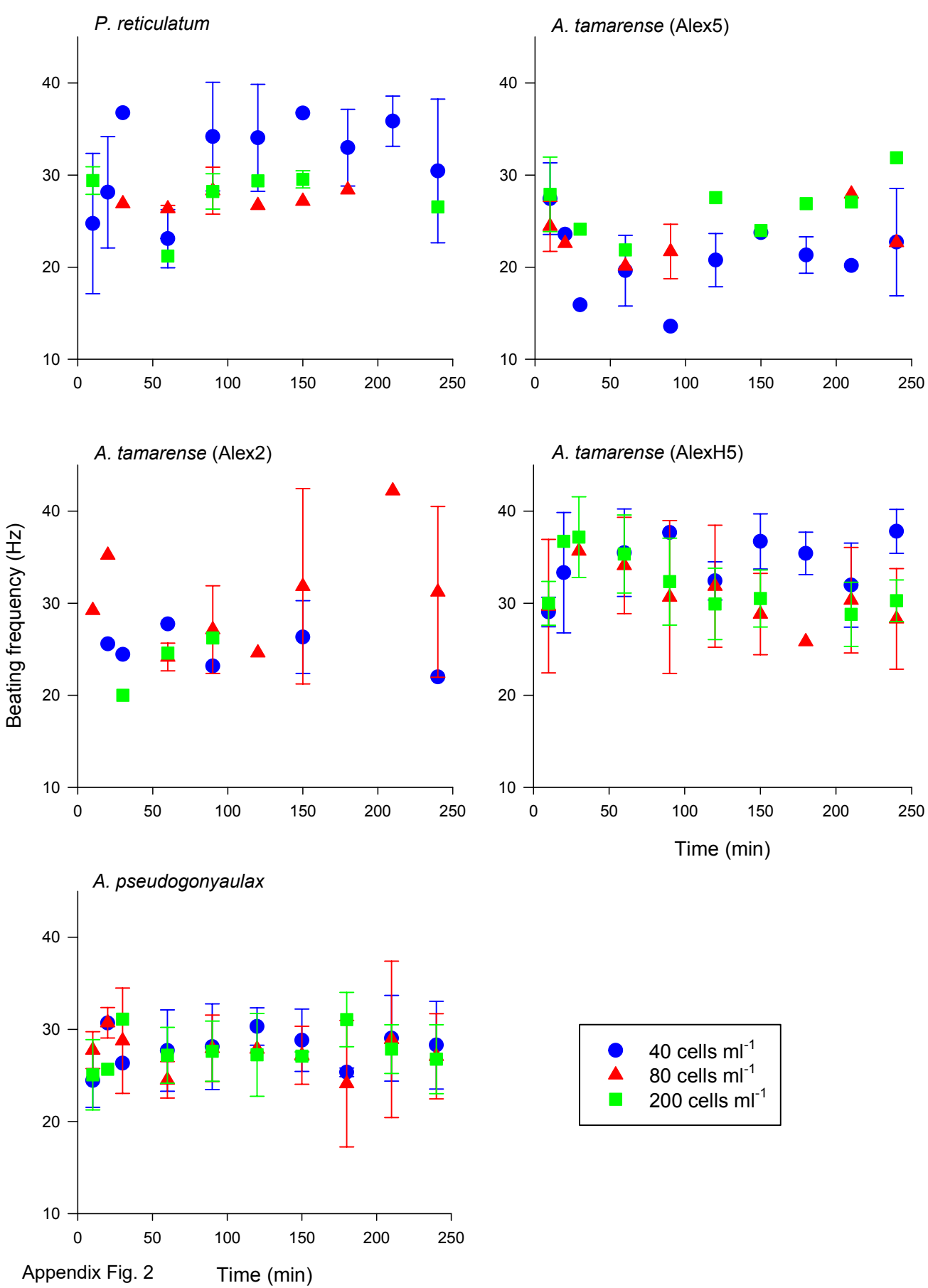


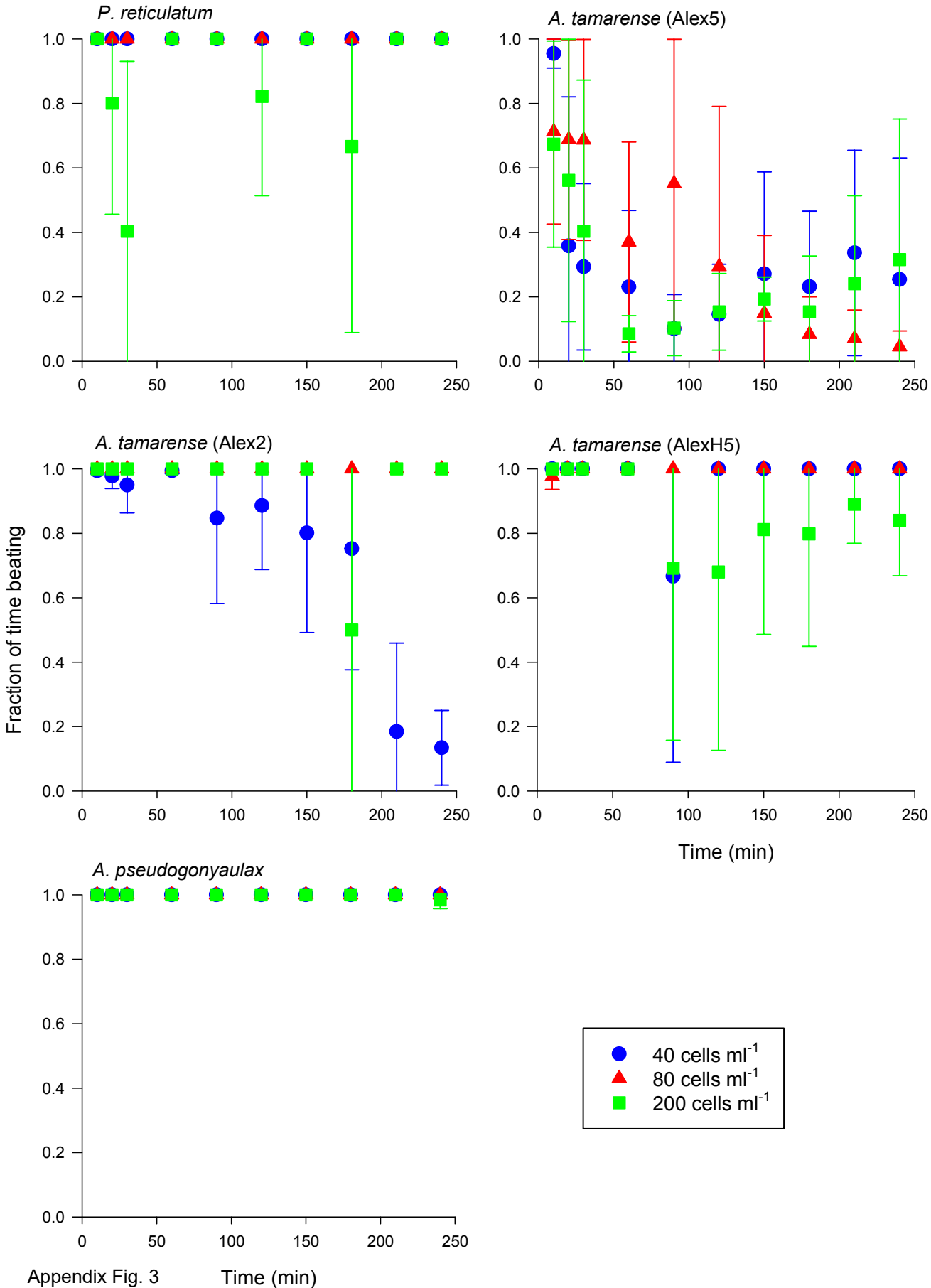
Fig.3

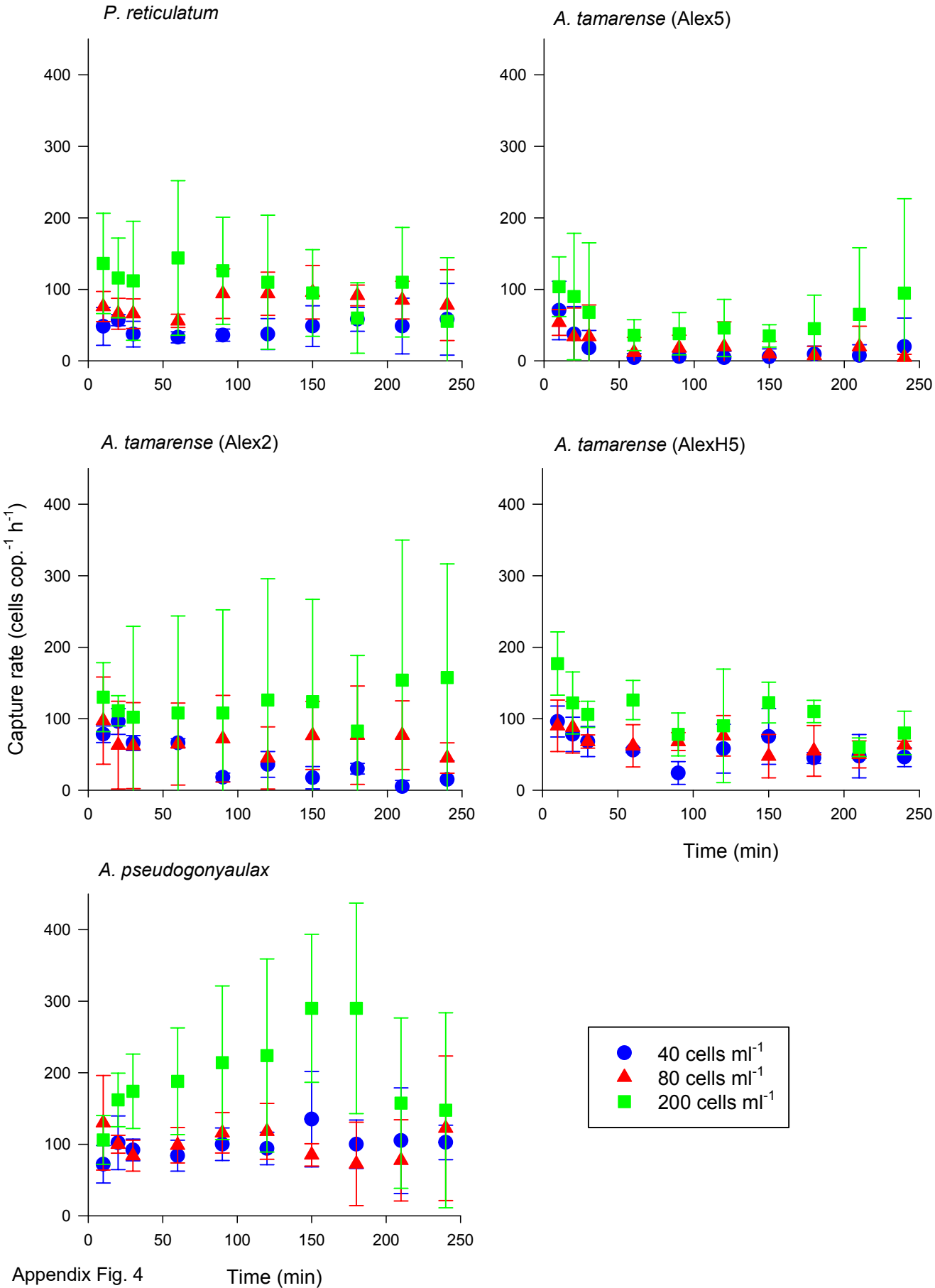


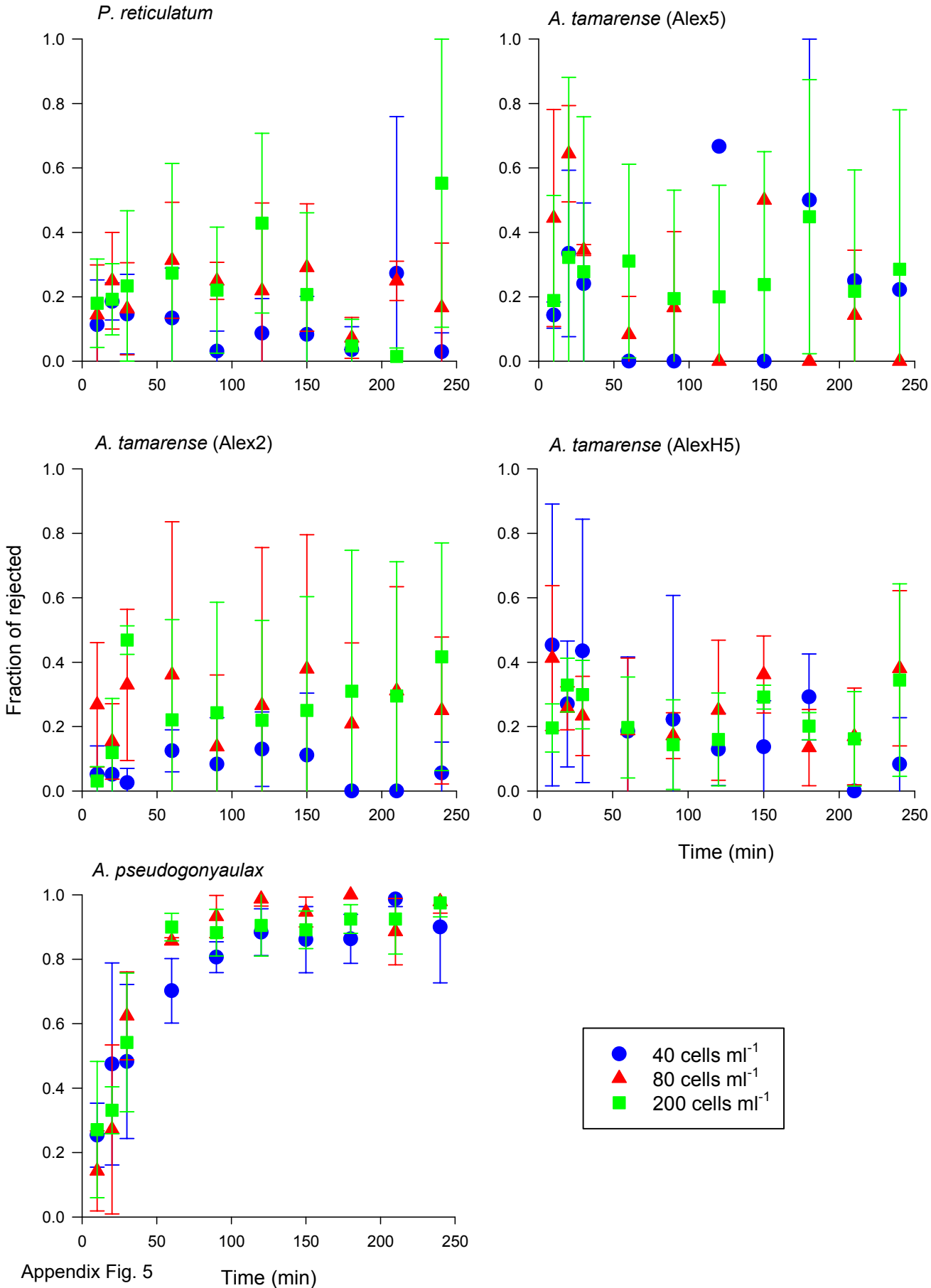
Appendix Fig.1

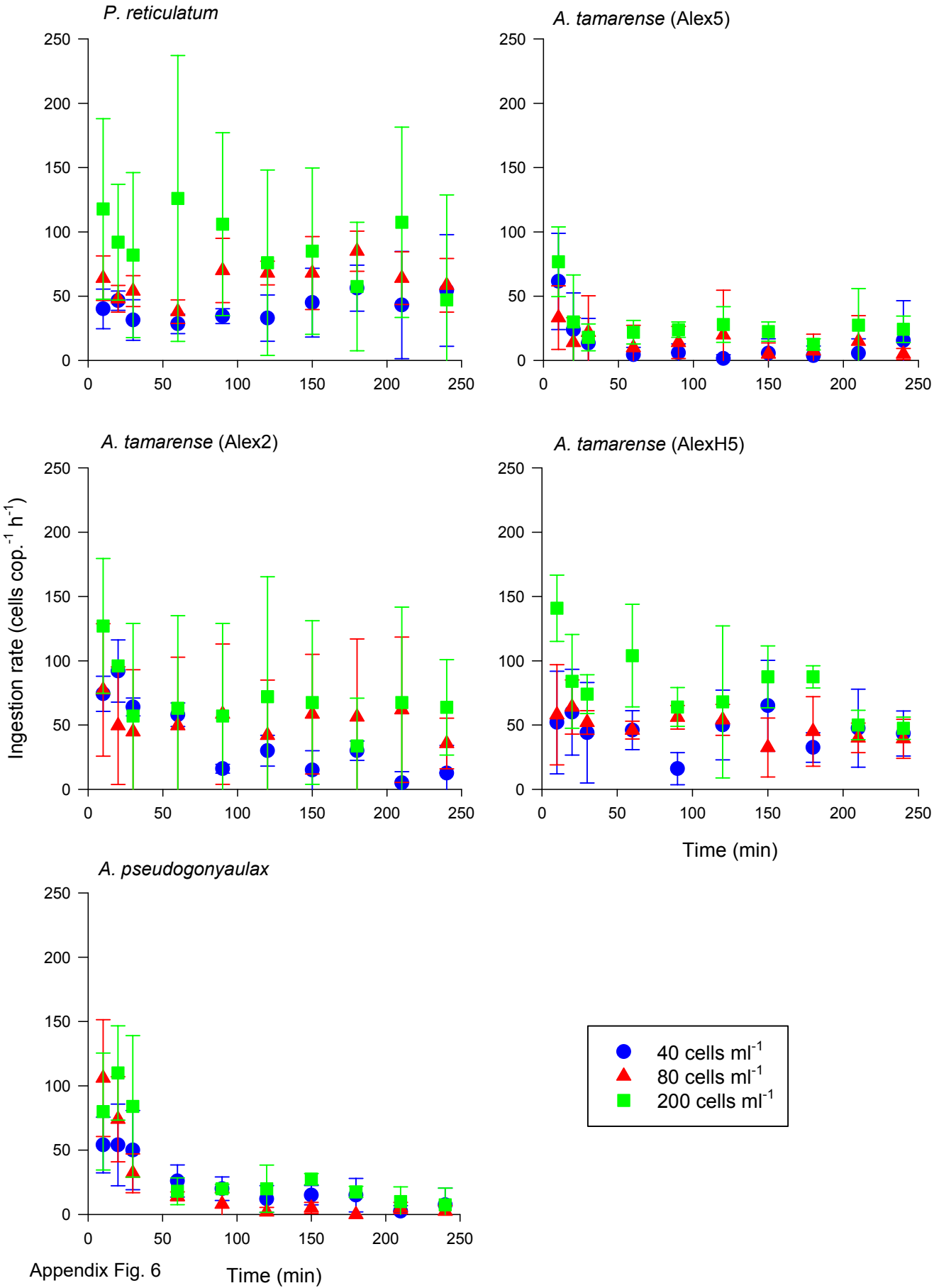




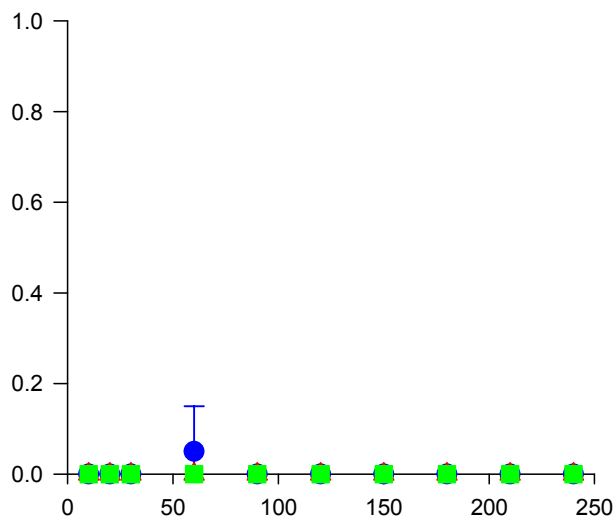




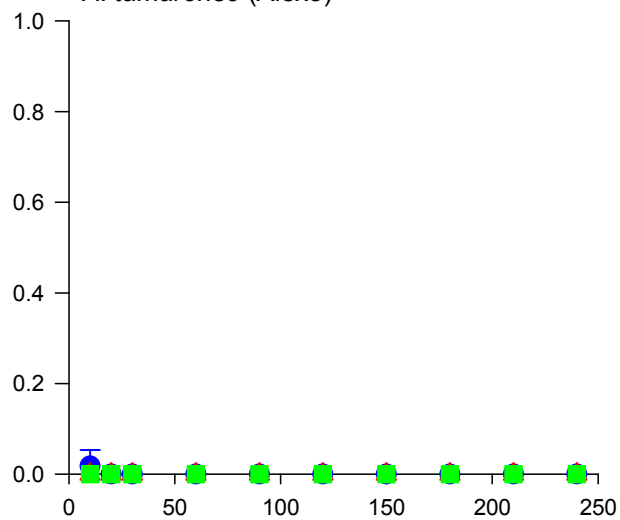




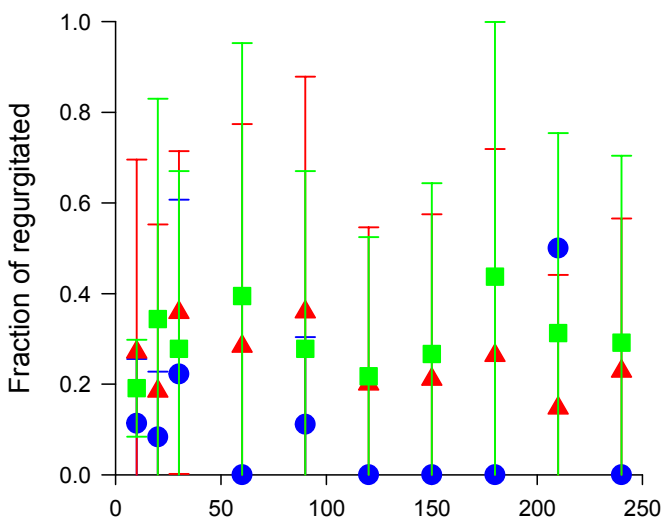
*P. reticulatum*



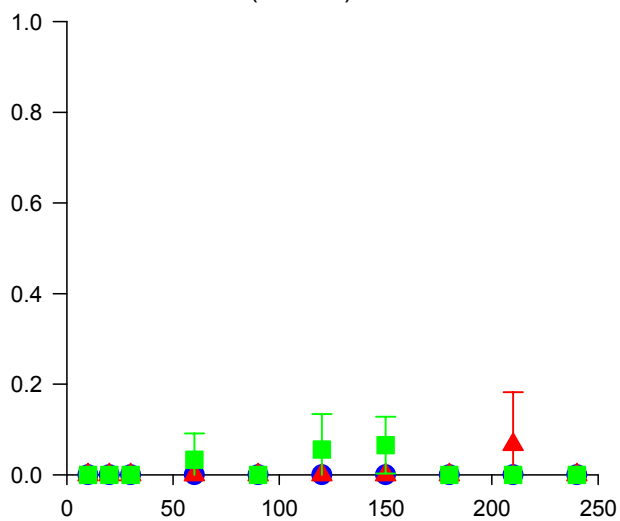
*A. tamarensis* (Alex5)



*A. tamarensis* (Alex2)

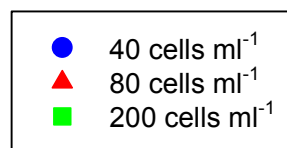
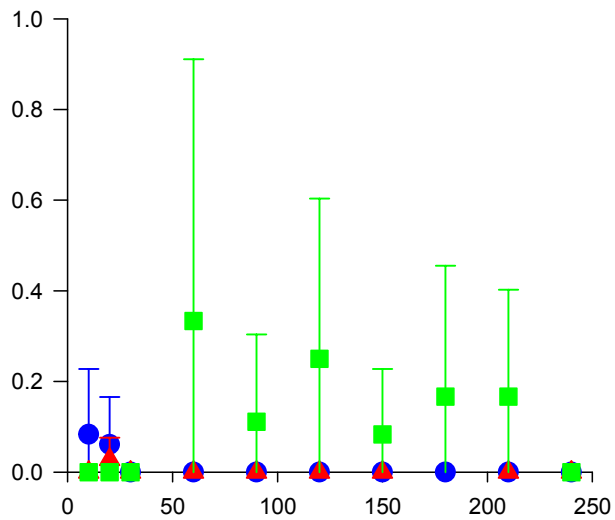


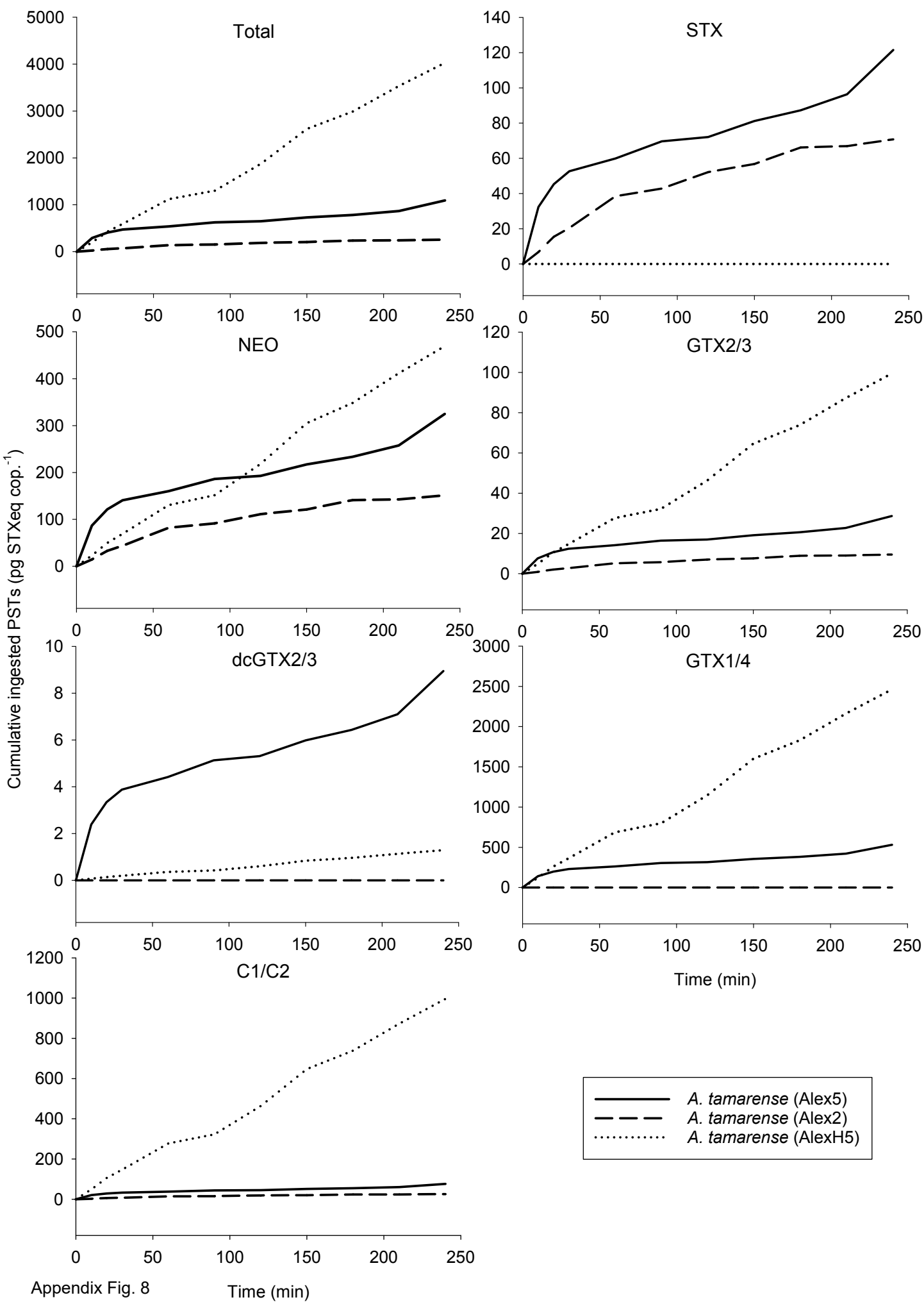
*A. tamarensis* (AlexH5)



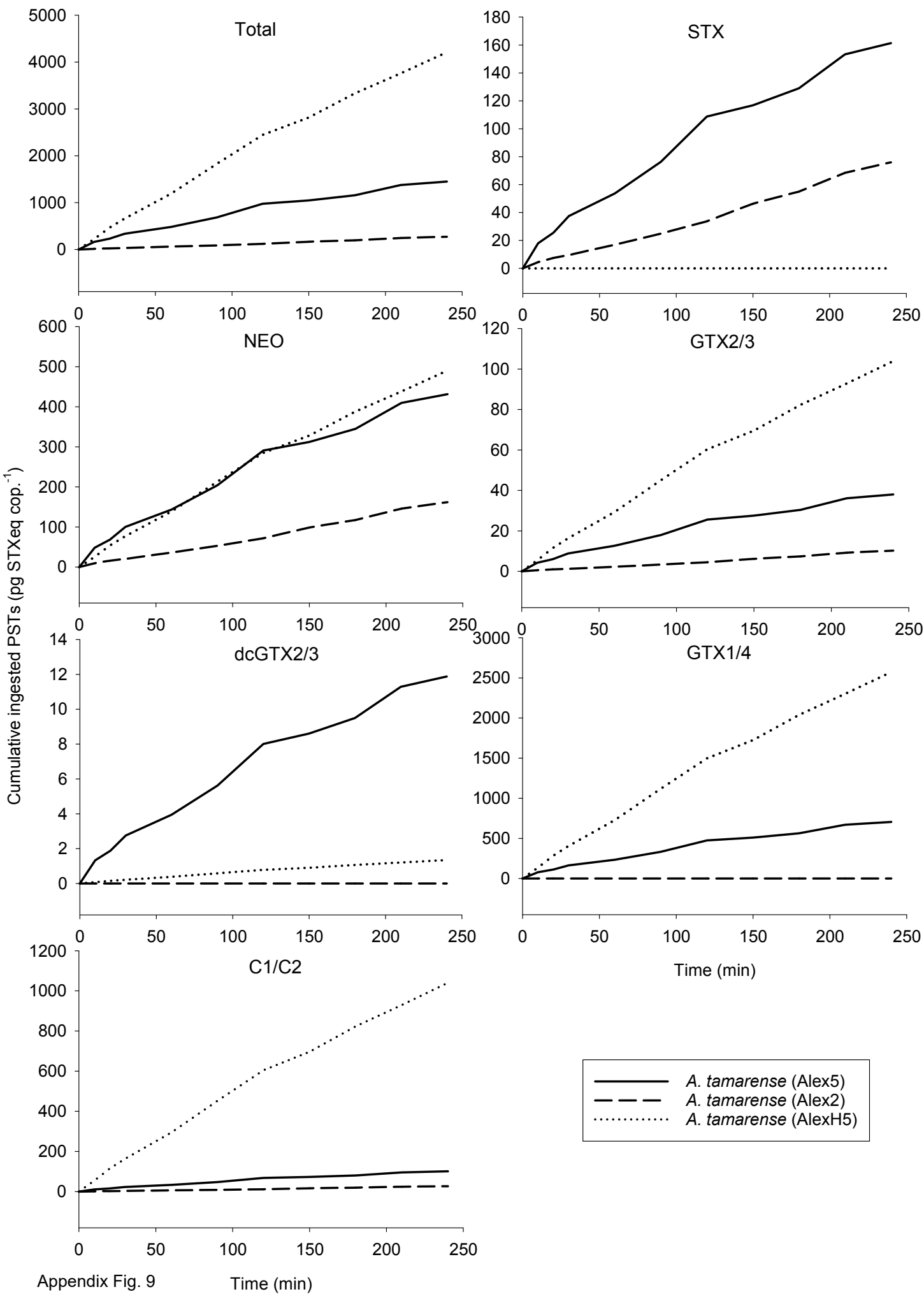
Time (min)

*A. pseudogonyaulax*

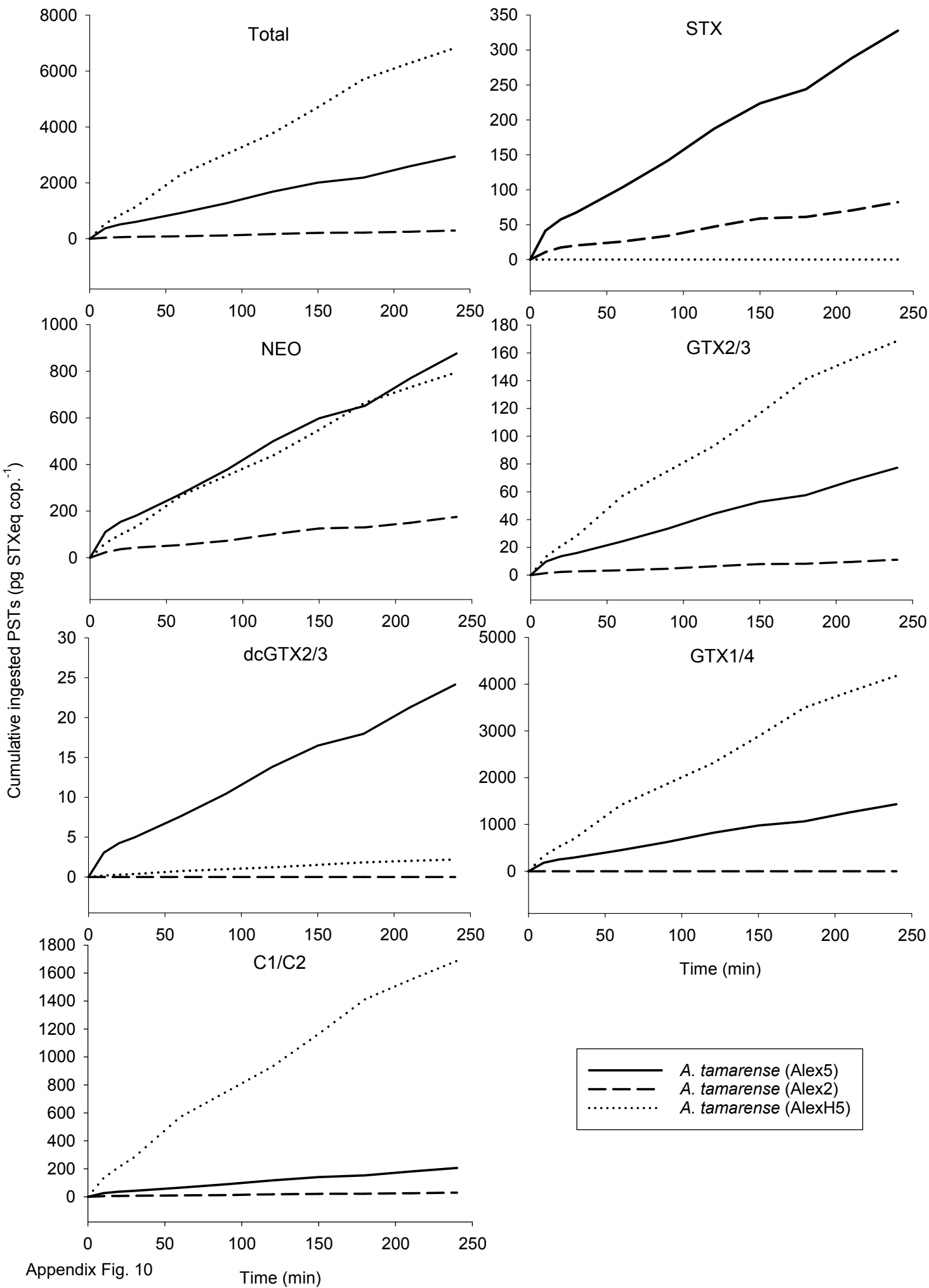




Appendix Fig. 8



Appendix Fig. 9



Appendix Fig. 10